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ACCELERATED COMMUNICATION

Cloning of a Novel Human Prostaglandin Receptor with Characteristics of the Pharmacologically Defined EP_2 Subtype

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SUMMARY

A cDNA that when expressed has the binding and functional characteristics of the pharmacologically defined EP_2 prostaglandin (PG) receptor [*Cardiovasc. Drug Rev.* 11:165–179 (1993)] has been cloned from a human placenta library. This clone, known as Hup-4, encodes a protein of 358 amino acids that has only ~30% overall identity with other PG receptors, including mouse and human clones that have been designated as EP_2 receptors [*J. Biol. Chem.* 268:7759–7762 (1993); *Biochem. Biophys. Res. Commun.* 197:263–270 (1993)]. In COS-7 cells transfected with Hup-4, PGE_2 stimulated the formation of cAMP with an EC_{50} of ~50 nM. The EP_2-selective agonists AH13205 and butaprost were also active, with EC_{50} values in the range of 2–6 μM. The order of potency of PGs for competition with binding of [^{3}H]PGE_2 to membranes prepared from COS-7 cells transfected with Hup-4 was $PGE_2 \geq PGE_1 >$ 16,16-dimethyl-$PGE_2 \geq$ 11-deoxy-$PGE_1 >$ butaprost > AH13205 > 19(*R*)-OH-PGE_2. Natural PGs and analogues that are selective for the FP ($PGF_{2\alpha}$), DP (PGD_2), EP_1 (sulprostone), EP_3 (MB 28767), and EP_4 (1-OH-PGE_1) receptors were inactive or competed poorly with the binding of [^{3}H]PGE_2 (<50% displacement of specific binding at 10 μM). Northern blot analysis showed the presence of a Hup-4 message of ~3.1 kilobases in mRNA from human lung and placenta. Reverse transcription-polymerase chain reaction studies also indicated that Hup-4 is probably expressed in human uterus and in HL-60 (human promyelocytic leukemia) cells. Our findings suggest that Hup-4 encodes the pharmacologically defined EP_2 receptor, whereas the mouse and human cDNAs previously classified as EP_2 may represent another EP receptor subtype or the recently defined EP_4 subtype [*Prostaglandins* 47:151–168 (1994)].

The PGs are locally acting hormones that have a remarkable variety of physiological actions in nearly all mammalian tissues. They are derived from unsaturated fatty acids either enzymatically (1) or by free radical-catalyzed peroxidation (2, 3). The many, and frequently opposing, effects of the PGs result in part from the number of naturally occurring metabolites and in part from the number of receptors they act on. There are five physiologically predominant PGs, which are all formed from arachidonic acid, the most abundant C_{20} fatty acid in mammals. These five metabolites, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and thromboxane A_2, appear to produce their physiological effects by way of separate receptors, which have been defined as DP, EP, FP, IP, and TP, respectively (4). There is also further subdivision of the EP receptors into EP_1, EP_2, and EP_3 (4), and a fourth subtype, EP_4, has been recently defined (5).

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Like many hormone receptors, PG receptors are not abundant, which makes it difficult to characterize the receptors biochemically and hinders the identification of compounds that selectively interact with the individual receptor subtypes. The molecular cloning of the genes encoding these receptors can indicate the biochemical basis of PG receptor heterogeneity and may aid in understanding their pharmacology. As has been the case with other subfamilies of G protein-coupled receptors, this process of molecular cloning has revealed greater complexity than previously thought. Thus, in addition to the cloning of genes encoding the pharmacologically characterized TP (6, 7), FP (8, 9), and EP (10–14) receptors, there has been the cloning of a variety of alternative mRNA splicing variants of the EP_3 receptor (15–18). Further complexity has also been suggested by virtue of an expressed cDNA encoding a putative EP_2 receptor whose pharmacology was not entirely as expected (12).

PG receptors are members of the superfamily of G protein-coupled receptors that appear to have a single subunit structure containing seven membrane-spanning domains (19). It is be-

ABBREVIATIONS: PG, prostaglandin; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; kb, kilobase(s).

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         10          20          30          40          50          60          70          80          90         100
GGGCCGCCGT CGGCGCGCTG GGTGCGGGAA GGGGGCTCTG GATTTCGGTC CCTCCCCTTT TTCCTCTGAG TCTCGGAACG CTCCAGCTCT CAGACCCTCT
        110         120         130         140         150
TCCTCCCAGG TAAAGGCCGG GAGAGGAGGG CGCATCTCTT TTCCAGGCAC CCCACC

 160          170          180          190          200          210          220          230          240
ATG GGC AAT GCC TCC AAT GAC TCC CAG TCT GAG GAC TGC GAG ACG CGA CAG TGG CTT CCC CCA GGC GAA AGC CCA GCC ATC AGC
 M   G   N   A   S   N   D   S   Q   S   E   D   C   E   T   R   Q   W   L   P   P   G   E   S   P   A   I   S   28
                                                                                          transmembrane 1
      250          260          270          280          290          300          310          320
TCC GTC ATG TTC TCG GCC GGG GTG CTG GGG AAC CTC ATA GCA CTG GCG CTG CTG GCG CGC CGC TGG CGG GGG GAC GTG GGG TGC
 S   V   M   F   S   A   G   V   L   G   N   L   I   A   L   A   L   L   A   R   R   W   R   G   D   V   G   C   56

    330          340          350          360          370          380          390          400
AGC GCC GGC CGC AGG AGC TCC CTC TCC TTG TTC CAC GTG CTG GTG ACC GAG CTG GTG TTC ACC GAC CTG CTC GGG ACC TGC CTC
 S   A   G   R   R   S   S   L   S   L   F   H   V   L   V   T   E   L   V   F   T   D   L   L   G   T   C   L   84
                                    transmembrane 2
        420          430          440          450          460          470          480          490
ATC AGC CCA GTG GTA CTG GCT TCG TAC GCG CGG AAC CAG ACC CTG GTG GCA CTG GCG CCC GAG AGC CGC GCG TGC ACC TAC TTC
 I   S   P   V   V   L   A   S   Y   A   R   N   Q   T   L   V   A   L   A   P   E   S   R   A   C   T   Y   F   112
                                                                                          transmembrane 3
      500          510          520          530          540          550          560          570
GCT TTC GCC ATG ACC TTC TTC AGC CTG GCC ACG ATG CTC ATG CTC TTC GCC ATG GCC CTG GAG CGC TAC CTC TCG ATC GGG GCA
 A   F   A   M   T   F   F   S   L   A   T   M   L   M   L   F   A   M   A   L   E   R   Y   L   S   I   G   A   140

  580          590          600          610          620          630          640          650          660
CCC CTA CTT CTA CCA GCG CCG CGT CTC GCC TCC GGG GGC CTG GGC GTG CTG CCT GTC ATC TAT GCA GTC TCC CTG CTC TTC TGC
 P   L   L   L   P   A   P   R   L   A   S   G   G   L   G   V   L   P   V   I   Y   A   V   S   L   L   F   C   168
                                            transmembrane 4
        670          680          690          700          710          720          730          740
TCG CTG CCG CTG CTG GAC TAT GGG CAG TAC GTC CAG TAC TGC CCC GGG ACC TGG TGC TTC ATC CGG CAC GGG CGG ACC GCT TAC
 S   L   P   L   L   D   Y   G   Q   Y   V   Q   Y   C   P   G   T   W   C   F   I   R   H   G   R   T   A   Y   196

    750          760          770          780          790          800          810          820
CTG CAG CTG TAC GCC ACC CTG CTG CTG CTT CTC ATT GTC TCG GTG CTC GCC TGC AAC TTC AGT GTC ATT CTC AAC CTC ATC CGC
 L   Q   L   Y   A   T   L   L   L   L   L   I   V   S   V   L   A   C   N   F   S   V   I   L   N   L   I   R   224
          transmembrane 5
        840          850          860          870          880          890          900          910
ATG CAC CGC CGA AGC CGG AGA AGC CGC TGC GGA CCT TCC CTG GGC AGT GGC CGG GGC GGC CCC GGG GCC CGC AGG AGA GGG GAA
 M   H   R   R   S   R   R   S   R   C   G   P   S   L   G   S   G   R   G   G   P   G   A   R   R   R   G   E   252

      920          930          940          950          960          970          980          990
AGG GTG TCC ATG GCG GAG GAG ACG GAC CAC CTC ATT CTC CTG GCT ATC ATG ACC ATC ACC TTC GCC GTC TGC TCC TTG CCT TTC
 R   V   S   M   A   E   E   T   D   H   L   I   L   L   A   I   M   T   I   T   F   A   V   C   S   L   P   F   280
                                        transmembrane 6
 1000         1010         1020         1030         1040         1050         1060         1070         1080
ACG ATT TTT GCA TAT ATG AAT GAA ACC TCT TCC CGA AAG GAA AAA TGG GAC CTC CAA GCT CTT AGG TTT TTG TCA ATT AAT TCA
 T   I   F   A   Y   M   N   E   T   S   S   R   K   E   K   W   D   L   Q   A   L   R   F   L   S   I   N   S   308
                                                                              transmembrane 7
        1090         1100         1110         1120         1130         1140         1150         1160
ATA ATT GAC CCT TGG GTC TTT GCC ATC CTT AGG CCT CCT GTT CTG AGA CTA ATG CGT TCA GTC CTC TGT TGT CGG ATT TCA TTA
 I   I   D   P   W   V   F   A   I   L   R   P   P   V   L   R   L   M   R   S   V   L   C   C   R   I   S   L   336

   1170         1180         1190         1200         1210         1220         1230         1240
AGA ACA CAA GAT GCA ACA CAA ACT TCC TGT TCT ACA CAG TCA GAT GCC AGT AAA CAG GCT GAC CTT TGA GGTCAGT
 R   T   Q   D   A   T   Q   T   S   C   S   T   Q   S   D   A   S   K   Q   A   D   L   *                    358

      1250       1260       1270       1280       1290       1300       1310       1320       1330       1340
AGTTTAAAAG TTCTTAGTTA TATAGCATCT GGAAGATCAT TTTGAAATTG TTCCCTGGAG AAATGAAAAC AGTGTGTAAA CAAAATGAAG CTGCCCTAAT
      1350       1360       1370       1380       1390       1400       1410       1420       1430       1440
AAAAAGGAGT ATACAAACAT TTAAGCTGTG GTCAAGGCTA CAGATGTGCT GACAAGGCAC TTCATGTAAA GTGTCAGAAG GAGCTACAAA ACCTACCCTC
      1450       1460       1470       1480       1490       1500       1510       1520       1530       1540
AATGAGCATG GTACTTGGCC TTTGGAGGAA CAATCGGCTG CATTGAAGAT CCAGCTGCCT ATTGATTTAA GCTTTCCTGT TGAATGACAA AGTATGTGGT
      1550       1560       1570       1580       1590       1600       1610       1620       1630       1640
TTTGTAATTT GTTTGAAACC CCAAACAGTG ACTGTACTTT CTATTTTAAT CTTGCTACTA CCGTTATACA CATATAGTGT ACAGCCAGAC CAGATTAAAC
      1650       1660       1670       1680       1690       1700       1710       1720       1730       1740
TTCATATGTA ATCTCTAGGA AGTCAATATG TGGAAGCAAC CAAGCCTGCT GTCTTGTGAT CACTTAGCGA ACCCTTTATT TGAACAATGA AGTTGAAAAT
      1750       1760       1770       1780       1790       1800       1810       1820       1830       1840
CATAGGCACC TTTTACTGTG ATGTTTGTGT ATGTGGGAGT ACTCTCATCA CTACAGTATT ACTCTTACAA GAGTGGACTC AGTGGGTTAA CATCAGTTTT
      1850       1860       1870       1880       1890       1900       1910       1920       1930       1940
GTTTACTCAT CCTCCAGGAA CTGCAGGTCA AGTTGTCAGG TTATTTATTT TATAATGTCC ATATGCTAAT AGTGATCAAG AAGACTTTAG GAATGGTTCT
      1950       1960       1970       1980       1990       2000       2010       2020       2030       2040
CTCAACAAGA AATAATAGAA ATGTCTCAAG GCAGTTAATT CTCATTAATA CTCTTATTAT CCTATTTCTG GGGGAGGATG TACGTGGCCA TGTATGAAGC
      2050       2060       2070       2080       2090       2100       2110       2120       2130       2140
CAAATATTAG GCTTAAAAAC TGAAAAATCT GGTTCATTCT TCAGATATAC TGGAACCCTT TTAAAGTTGA TATTGGGGCC ATGAGTAAAA TAGATTTTAT
      2150       2160       2170       2180       2190       2200       2210       2220       2230       2240
AAGATGACTG TGTTGTACCA AAATTCATCT GTCTATATTT TATTTAGGGG AACATGGTTT GACTCATCTT ATATGGGAAA CCATGTAGCA GTGAGTCATA
      2250       2260       2270       2280       2290          ↓       2310       2320       2330       2340
TCTTAATATA TTTCTAAATG TTTGGCATGT AAATGTAAAC TCAGCATCAA AATATTTCAG TGAATTTGCA CTGTTTAATC ATAGTTACTG TGTAAACTCA
      2350       2360       2370       2380
TCTGAAATGT TACAAAAATA AACTATAAAA CAAAAAAAAA
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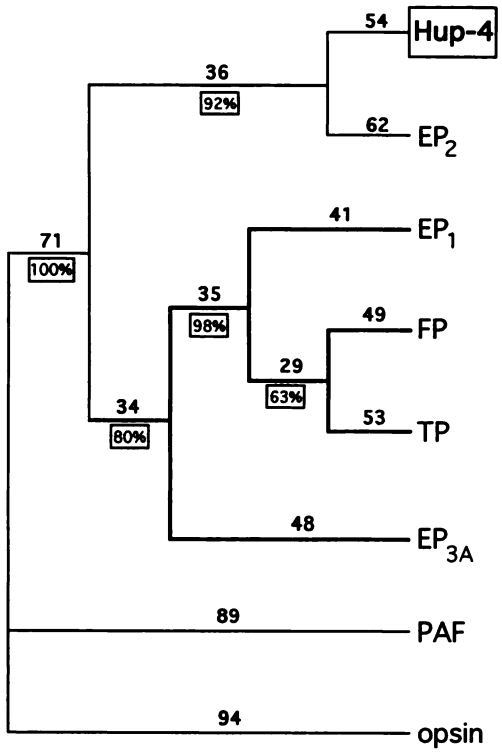

Fig. 2. Phylogeny of the human PG and platelet-activating factor (*PAF*) receptors, based upon partial alignment of their protein sequences and the algorithms of Swofford (27), involving protein parsimony. Alignment of the deduced amino acid sequences of Hup-4 and the EP_1 (11), EP_2 (13), EP_{3A} (17), FP (9), TP (6), and platelet-activating factor (38) receptors was done using human opsin (39) as the outgroup. The alignments were done maximizing homology, using all of the transmembrane domains and the region between transmembrane region 4 and transmembrane region 5. The most parsimonious phylogeny (which is shown) had a total of 695 steps and a consistency index of 0.891. Branch lengths are *bold* and represent the number of steps or changes a group has undergone. Bootstrap analysis was done by random step addition with 20 replications/step and 100 total steps. Bootstrap values are *boxed* and represent the percentage of times the grouping to the right occurred within the analysis.

lieved that ligand binding takes place in the membrane-spanning domains and that G protein coupling occurs through the intracellular loops and carboxyl terminus. In the case of the mRNA splicing variants of the EP_3 receptor, it has been found that the carboxyl terminus is also important for the desensitization of these receptors (20) and for specifying their interaction with G proteins (15, 16). Although the cloned PG receptors share common motifs in their deduced amino acid sequences, the overall sequence identity between the different subtypes is only on the order of 30%. This is even the case within a subdivision; thus, the differences between EP_1 and EP_3 receptors are nearly as great as those between EP_1 and FP receptors or between EP_1 and TP receptors.

To further understand the molecular structure and classification of the PG receptors, we were interested in cloning additional members of this receptor family. Using a cDNA probe encoding the human EP_{3A} receptor (17), we screened a library from human placenta and isolated a cDNA clone that encoded a related PG receptor. Expression of this receptor showed that it stimulated cAMP formation and had an EP_2 pharmacology. Its amino acid identity with two previously cloned receptors, also designated as EP_2 (12, 13), was only ~30%, suggesting that these previously cloned receptors might represent another EP receptor subtype.

Materials and Methods

cDNA cloning. A cDNA containing the complete coding sequence of the human EP_{3A} receptor (17) was labeled with ^{32}P by nick-translation (GIBCO/BRL) and was used to screen a human placenta cDNA library (Clontech) by plaque hybridization analysis (21). The library, constructed in λgt11, was plated at a density of ~25,000 plaques/plate (15 cm) using $Y1090R^-$ cells. A total of 16 plates were used, from which impressions were taken using nylon membranes (Colony Plaque Screen; DuPont). The membranes were denatured, baked at 80° for 2 hr, and prehybridized for 2 hr at 37° in 50% deionized formamide, 1% sodium dodecyl sulfate, 1 M NaCl, 100 μg/ml sonicated/boiled herring sperm DNA. The probe was added at a concentration of ~5×10^6 dpm/ml and was hybridized with the membranes overnight at 37°. The membranes were washed for 1 hr at 45° in 1× standard saline citrate 150 mM NaCl/0.1% sodium dodecyl sulfate and were placed in cassettes for overnight exposure to Kodak XAR film at −70°.

Using the PCR and restriction enzyme analysis, seven related clones were identified and could be put into three groups on the basis of their size. One member from each of the groups was subcloned into the *Eco*RI site of pBluescript (Stratagene) and its nucleotide sequence was determined using the dideoxy-chain termination method (Sequenase; United States Biochemical). All of the clones were found to contain overlapping sequences, and one clone, Hup-4, contained a complete open reading frame of 1074 nucleotides.

Construction of an expression vector. A plasmid for expressing Hup-4 in eukaryotic cells was made as follows. PCR was used to amplify a portion of the 5′ end of KS/Hup (the pBluescript clone of Hup-4) containing a unique *Sac*I site (nucleotides 339–343). The sense primer represented nucleotides 124–147 (underlined type) and contained additional *Sac*I (bold type) and *Dra*I (lowercase letters) restriction enzyme sites, as follows: 5′-GATGAGCTCtttaaaAGGAGGGCGCATCTCTTTTCCAGG-3′. The antisense primer represented nucleotides 370–387. The PCR product was digested with *Sac*I and was ligated to the large fragment remaining from the digestion of KS/Hup with *Sac*I. *Escherichia coli* cells were transformed and a plasmid with the correct orientation of the PCR insert was isolated. The latter was digested with *Dra*I and was ligated to pBC12BI (22), which had been cleaved with *Bam*HI and *Hind*III and filled in with Klenow. *E. coli* cells were transformed and a plasmid, pBC/Hup, was isolated in which the *Dra*I site adjacent to nucleotide 124 in Hup-4 was ligated to the *Hind*III site (nucleotide 314 in pBC12BI).

Expression and radioligand binding. COS-7 cells were transfected with pBC/Hup using DEAE-dextran and dimethylsulfoxide shock (22). For radioligand binding, the cells were harvested after 3 days and membranes were prepared (23), resuspended at a concentration of 1 mg/ml in 50 mM Tris·HCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.4, and frozen at −80°. The membrane suspensions were thawed at room temperature and were used in a 200-μl binding assay at a final concentration of ~500 μg/ml. The binding of [^{3}H]PGE_2 (specific activity, 183 Ci/mmol; Amersham) was determined in duplicate and the

Fig. 1. Nucleotide and deduced amino acid sequences of Hup-4, a human placenta cDNA encoding a PG EP_2 receptor subtype. Clones were isolated and sequenced as described in Materials and Methods. Putative transmembrane domains are indicated and were determined by hydropathy analysis and consensus with other cloned PG receptors. The nucleotide sequence of Hup-4 ended with nucleotide 2296, and the additional 3′ sequence was provided by clones Hup-2 (nucleotides 618–2323) and Hup-3 (nucleotides 345–2380). *Sac*I and *Dra*I sites used in constructing the expression plasmid and in making a radiolabeled probe are *underlined*.

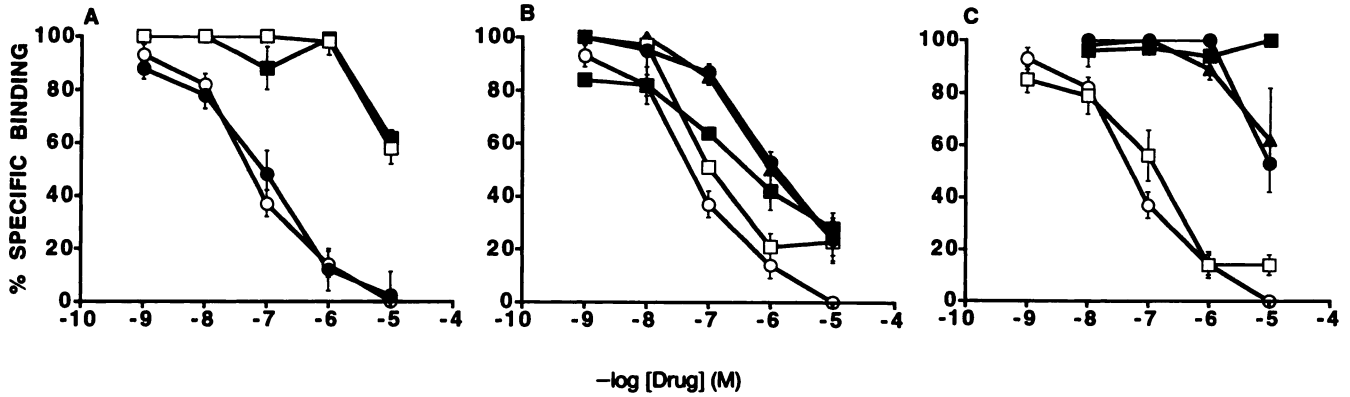

Fig. 3. Competition by PGs with the radioligand binding of [^{3}H]PGE_2 to membranes prepared from COS-7 cells transfected with pBC/Hup. A, Naturally occuring PGs. ○, PGE_2; ●, PGE_1; □, PGD_2; ■, $PGF_{2\alpha}$. B, Reported EP_2-selective PGs. ▲, AH13205; ■, butaprost; ●, 19(*R*)-OH-PGE_2; □, 11-deoxy-PGE_1. C, Other PGs. □, 16,16-Dimethyl-PGE_2; ▲, MB28767; ■, sulprostone; ●, 1-OH-PGE_1. In B and C the data from A for PGE_2 (○) are added for comparison. Transfections and radioligand binding were done as described in Materials and Methods. Data are the means ± standard errors of three separate experiments performed in duplicate.

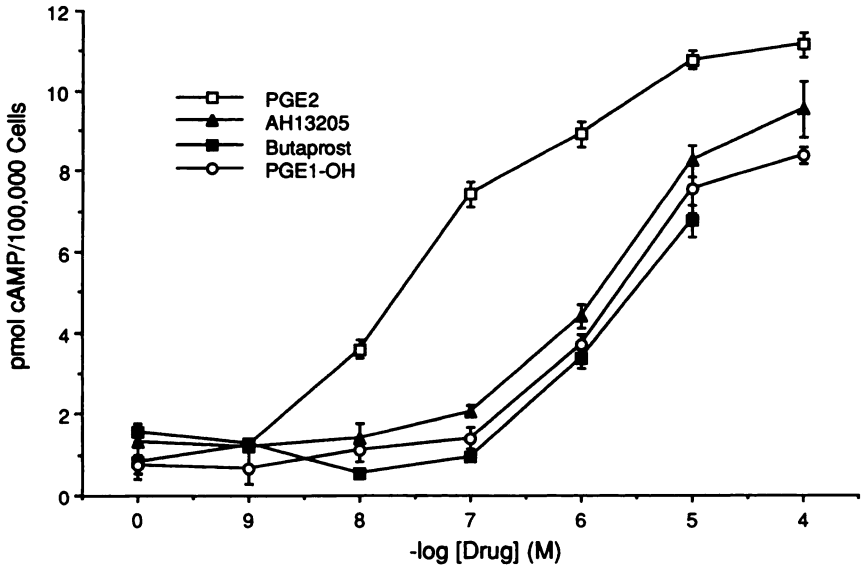

Fig. 4. Stimulation by PGs of cAMP formation in COS-7 cells transfected with pBC/Hup. Transfections and cAMP determinations were done as described in Materials and Methods. The data are from one experiment that was replicated three times. Each *point* is the average of two wells of transfected cells. EC_{50} values (mean ± standard error) for the three experiments are as follows: PGE_2, 43 ± 6 nM; butaprost, 5800 ± 400 nM; AH13205, 3100 ± 1000 nM; 1-OH-PGE_1, 2000 ± 1800 nM.

experiments were replicated three times. Incubations were for 30 min at 25° and were terminated by the addition of 4 ml of ice-cold 50 mM Tris·HCl, followed by rapid filtration through Whatman GF/B filters and three additional 4-ml washes with a cell harvester (Brandel). Competition studies were done with a final concentration of 5 nM [^{3}H] PGE_2, and nonspecific binding was determined with a final concentration of 10 μM PGE_2. Data were analyzed by computer using the EBDA/LIGAND program (24).

Expression and cAMP determination. A cAMP binding assay utilizing protein kinase A (25) was used to study the effects of PGs on cAMP formation in COS-7 cells transfected with pBC/Hup. Cells were transfected as described above and 24 hr later they were split into 24-well plates and cultured for 2 more days in DMEM containing 5% bovine calf serum. The cells were rinsed with DMEM and preincubated for 1 min at 37° with 400 μl/well DMEM containing 100 μg/ml isobutylmethylxanthine. The cells were then incubated for 3 min at 37° after the addition of 100 μl/well DMEM containing isobutylmethylxanthine and the desired final drug concentration. The medium was removed and the cells were scraped in 150 μl/well ice-cold TE (50 mM Tris, 4 mM EDTA, pH 7.5) and placed on ice. The cell suspensions were transferred to microcentrifuge tubes, boiled for 5 min, and frozen at −20°.

For cAMP determination, the samples were thawed and centrifuged, and 50 μl were removed and added to 26 nCi of [^{3}H]cAMP (28.9 Ci/mmol; NEN/DuPont) in 50 μl of TE. Protein kinase A (P-5511; Sigma) was dissolved at a concentration of 60 μg/ml in TE containing 0.1% bovine serum albumin, and 100 μl were added to each of the samples. The samples were incubated for 2 hr on ice, and 100 μl of TE containing 2% bovine serum albumin and 26 mg/ml activated charcoal were added. The samples were centrifuged and the radioactivity in 200 μl of the supernatant was compared with a standard curve containing 0.125–64 pmol of cAMP/50-μl sample (see above).

PCR and Northern blotting. PCR and Northern blot analysis were done as described previously, using the probes and primers relevant to the present studies (17). For PCR analysis of the cDNA clones, primers specific for the λgt11 sequence were used (Clontech). For reverse transcription-PCR, primers corresponding to the second extracellular loop (nucleotides 740–757) and to transmembrane region 7 (nucleotides 1090–1107) were used. For Northern blot analysis, probes representing the entire Hup-4 cDNA and a *Dra*I fragment (nucleotides 1246–2102) were used. The probes were nick-translated with ^{32}P (GIBCO/BRL) and were used at a concentration of ~1.5 × 10^6 dpm/ml. The Northern blot was prepared as recommended by the manufacturer (7760–1; Clontech) and contained ~2 μg/lane poly(A)$^+$ RNA.

Results

A cDNA containing the full coding sequence of the human EP_{3A} receptor (17) was radiolabeled with ^{32}P and was used to

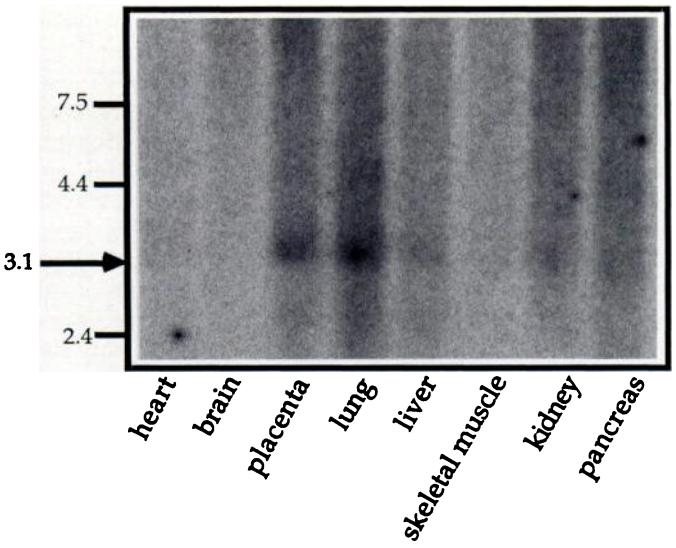

Fig. 5. Northern blot analysis of mRNA from human tissues. Preparation of the probe and hybridization conditions were as described in Materials and Methods. The probe was labeled with ^{32}P and represented a *Dra*I fragment from the 3′ untranslated region of Hup-4. Approximately 2 μg of mRNA were loaded in each lane. The blot was stripped and reprobed with ^{32}P-labeled actin cDNA, showing that a similar amount of actin message was present in each lane. Numbers and arrows refer to kilobases (Kb)

screen a human placenta cDNA library under conditions of low stringency. From a total of 400,000 recombinants, seven clones were obtained and were divided into three groups on the basis of their sizes and restriction enzyme analyses. Clones from each group were sequenced and one, Hup-4, was found to contain a complete open reading frame of 1074 nucleotides. The composite nucleotide and deduced amino acid sequences of the clones are shown in Fig. 1. The deduced amino acid sequence of Hup-4 encodes a protein of 358 amino acids (M_r 39,380) with seven hydrophobic regions that could represent the membrane-spanning domains of the receptor. In the hydrophilic regions of the deduced sequence there are four consensus sites for *N*-linked glycosylation, i.e., two in the amino terminus and one each in the proposed first and third extracellular loops. Whether this receptor is glycosylated *in vivo*, however, is presently unknown.

An amino acid alignment of Hup-4 with other G protein-coupled receptors show that Hup-4 is a novel receptor that has its greatest amino acid identity with other members of the family of prostanoid receptors (data not shown). Within the proposed transmembrane domains, its amino acid identity with other cloned PG receptors is as follows: EP_1, 37%; EP_2, 38%; EP_{3A}, 34%; TP, 31%; FP, 31%. Certain regions that have been previously identified as being well conserved in PG receptors are also conserved in Hup-4. These include such sequences as LxxTDxxG in transmembrane region 2, QYxPGxWCF in the second extracellular loop, and NxIxDPW in transmembrane region 7. Two amino acids downstream of the latter sequence is an interesting change in which a tyrosine that is present in nearly all G protein-coupled receptors is a phenylalanine in Hup-4. This position has recently been shown to be important in the process of receptor sequestration (26).

To further explore the relationship of Hup-4 to the other previously cloned PG receptors, a phylogeny was created using the protein sequence parsimony methods of Swofford (27). The results of this analysis, displayed in Fig. 2, show two major groups within the PG receptor family. One group consists of Hup-4 and the previously cloned putative EP_2 receptor, whereas the other group consists of the EP_1, EP_3, FP, and TP receptors. Within the latter group, the EP_3 receptors are distinct, in that they diverged as a separate branch before the divergence of the EP_1, FP, and TP receptors. Bootstrap analysis, a statistical method that indicates the number of times a particular grouping occurred within the analysis, gave high values to the grouping of Hup-4 with the previously cloned putative EP_2 receptor (92%). High bootstrap values were also obtained for the grouping of the EP_1, EP_3, FP, and TP receptors (80%) and for the subgrouping of the EP_1, FP, and TP receptors (98%). A lower value, however, was obtained for the subgrouping of the FP and TP receptors (63%), which indicates that phylogenetic relationships within the EP_1, FP, and TP receptors cannot be resolved with confidence.

The pharmacology of Hup-4 was characterized by [^{3}H]PGE_2 binding after transient expression in COS-7 cells. Fig. 3 shows the results of competition analyses using a variety of PG analogues. Of the naturally occurring PGs, PGE_2 and PGE_1 clearly had the highest affinity (Fig. 3A), suggesting that Hup-4 is a member of the EP family of PG receptors. Further analysis, in fact, showed that the pharmacology of Hup-4 was very much like that of the pharmacologically defined EP_2 receptor (4). For example, AH13205 and butaprost, synthetic PG agonists with selectivity for the EP_2 subtype (5, 28–32), showed complete dose-dependent inhibition of [^{3}H]PGE_2 binding to Hup-4 (Fig. 3B). In addition, 19(*R*)-OH-PGE_2 and 11-deoxy-PGE_1, two other PGs with reported selectivity for the EP_2 subtype (33, 34), also competed with the binding of [^{3}H]PGE_2 to Hup-4 (Fig. 3B). On the other hand, 1-OH-PGE_1, a PG with selectivity for the EP_4 subtype,[1] and sulprostone, a PG with selectivity for the EP_1 and EP_3 subtypes, competed poorly with the binding of [^{3}H]PGE_2 to Hup-4 (Fig. 3C).

The second messenger pathway utilized by EP_2 receptors involves the activation of adenylyl cyclase (4, 28); therefore, PG-stimulated cAMP formation was examined in COS-7 cells transiently transfected with Hup-4. Fig. 4 shows that PGE_2 could potently stimulate the formation of cAMP, followed by AH13205, 1-OH-PGE_1, and butaprost. The results of three experiments indicated that the relative potencies of AH13205 and 1-OH-PGE_1 could not be distinguished, whereas that of butaprost appeared slightly lower. The possibility that 1-OH-PGE_1 was being oxidized to PGE_1, thereby increasing the apparent potency of 1-OH-PGE_1, was not investigated. Control studies with mock-transfected COS-7 cells showed no specific binding of [^{3}H]PGE_2 or PGE_2-stimulated cAMP formation (data not shown). In addition, PGE_2 did not stimulate phosphoinositide hydrolysis in COS-7 cells transiently transfected with Hup-4.

Northern blot analysis was used to examine the tissue distribution of Hup-4, and the results are shown in Fig. 5. Using a ^{32}P-labeled probe derived from the 3′ untranslated region of Hup-4, a message of ~3.1 kb was detected in mRNA from human placenta and lung. The mRNA from heart, brain, and skeletal muscle appeared negative, whereas the results obtained for kidney, pancreas, and liver were inconclusive. A separate blot containing the same samples was hybridized with the

[1] J. Chen, C.E. Protzman, and D.F. Woodward, unpublished observations.

complete Hup-4 cDNA and the same results were obtained, except for a higher background level (data not shown).

Discussion

Hup-4, a cDNA encoding a novel PG receptor from human placenta, has been cloned, and it has the radioligand binding and functional characteristics of the pharmacologically defined EP_2 subtype (4, 28). The cloning of mouse (12) and human (13) EP_2 receptors has been reported previously but the deduced amino acid sequences of these clones have only ~30% overall identity with the deduced sequence of the present clone. This degree of identity is much lower than one would expect for a species homologue or an isoform. For example, the degree of identity between the two previously cloned EP_2 receptors is 88% and for the mouse EP_3 receptor isoforms is ~90% (15). An EP_4 receptor that has pharmacological similarities to the EP_2 subtype has been recently characterized (5). Evidence discussed below suggests that Hup-4 is a human EP_2 receptor subtype and that the previously cloned receptors are either an additional EP receptor subtype or the mouse and human EP_4 subtypes.

Until recently, the subfamily of PG EP receptors was considered to have three subtypes, i.e., EP_1, EP_2, and EP_3 (4). Rodent and human cDNA clones encoding all of these subtypes have been described and, in general, the pharmacological and functional characteristics of the expressed receptors have matched what was expected. However, discrepancies were noted in the pharmacology of the putative mouse EP_2 receptor (12). Thus, butaprost, an agonist known to be EP_2 selective in isolated smooth muscle preparations (30–32), did not compete with the binding of [^{3}H]PGE_2. The authors attributed this to possible species differences between EP_2 receptors or to the possible existence of other EP_2 receptor subtypes. Although a human subtype was cloned (13), these issues of species differences or additional subtypes could not be addressed because the pharmacological analysis was limited to four compounds (PGE_1, PGE_2, $PGF_{2\alpha}$, and PGD_2). The recent characterization of an EP_4 subtype (5) and the present cloning of Hup-4, however, offer an explanation.

The pharmacology of Hup-4, as determined by radioligand binding competition and stimulation of cAMP formation, is more consistent with EP_2 receptor pharmacology than the pharmacology of the previously cloned mouse EP_2 receptor (12). For example, the receptor encoded by Hup-4 binds [^{3}H]PGE_2 with high affinity and butaprost competes with this binding with an affinity consistent with its functional activity at EP_2 receptors in respiratory, vascular, and uterine smooth muscle (30, 35, 36). Similarly, other reported EP_2-selective compounds, such as 19(R)-OH-PGE_2 (33) and 11-deoxy-PGE_1 (34), also competed effectively with [^{3}H]PGE_2 for binding to Hup-4. AH13205, another EP_2-selective compound (28) that has been characterized as being inactive at EP_4 receptors (5), competed with moderate affinity for Hup-4. 1-OH-PGE_1, a PG analogue that has high potency for the EP_4 receptor in rabbit jugular vein but only weak activity for the EP_2 receptor in cat trachea,[2] was among the least effective compounds in competing with the binding of [^{3}H]PGE_2 to Hup-4. Neither 1-OH-PGE_1 nor AH13205 was included in the characterization of the putative mouse EP_2 receptor, so it is premature to conclude whether this mouse EP receptor represents EP_4 or yet another EP receptor subtype.

Consistent with the known functional coupling of EP_2 receptors to adenylyl cyclase, PGE_2 stimulated the formation of cAMP in COS-7 cells transiently transfected with Hup-4. Butaprost and AH13205 also stimulated cAMP formation, as did 1-OH-PGE_1. The activity of 1-OH-PGE_1, however, may have been in part due to oxidation of 1-OH-PGE_1 to PGE_1. Thus, at a concentration of 10 μM 1-OH-PGE_1, only 5% oxidation would yield enough PGE_1 to give nearly maximal stimulation of cAMP formation. Regarding the previously cloned receptors designated as EP_2 (12, 13), only PGE_2 was examined for its ability to stimulate cAMP formation. In both cases PGE_2 did stimulate cAMP formation, although the fold stimulation was lower than that obtained in the present studies with Hup-4.

Northern blot analyses also support an EP_2 classification for Hup-4 and show a pattern of hybridization that is quite distinct from that obtained for the cloned receptors previously designated as EP_2. For example, using Hup-4 as a probe, a message of ~3.1 kb was detected in mRNA from human placenta, whereas a corresponding message could not be detected when the previously cloned human EP_2 cDNA was used as a probe (13). In contrast, the previously cloned human EP_2 cDNA hybridized to a ~3.8-kb message in mRNA from human heart and skeletal muscle; however, a message was not detected when the same mRNA was probed with Hup-4. Both probes hybridized to mRNAs from human lung, a tissue thought to contain EP_2 receptors (28).

Additional studies with reverse transcription-PCR showed Hup-4 message in other tissues. Using primers corresponding to the second extracellular loop and transmembrane region 7, products of the appropriate size (368 nucleotides) were obtained from total RNA isolated from human uterus and HL-60 cells (data not shown). These primers did not yield a product with human genomic DNA, indicating that mRNA was the likely template for the products from human uterus and HL-60 cells and suggesting the presence of an intron in the genomic sequence. The hypothesis that the Hup-4 message in lung, HL-60 cells, and uterus encodes an EP_2 receptor is consistent with pharmacological studies of these tissues. Thus, the EP_2-selective agonist AH13205 inhibits the release of inflammatory mediators from human lung and neutrophils (28), and in human myometrium both AH13205 and the EP_2-selective agonist butaprost can inhibit contraction and stimulate cAMP formation (31, 36, 37).

Alignment of the amino acid sequence of Hup-4 with those of the other cloned human PG receptors, followed by analysis of the possible phylogeny of the PG receptor family, has yielded clues about the possible evolution of these receptors. First, it shows that Hup-4 and the previously cloned receptor designated EP_2 can be grouped together and, therefore, share a closer evolutionary relationship to each other than to the other PG receptors (EP_1, EP_3, FP, and TP), which form a separate group. The fact that receptors with an EP pharmacology are present in each of these major groups is interesting and suggests that the ancestral receptor was EP. It also suggests that the receptors diverged initially along functional lines and then with respect to ligand binding. Thus, Hup-4 and the previously cloned receptor designated as EP_2 both stimulate adenylyl cyclase, whereas the other PG receptors either inhibit cyclase or stimulate phosphatidylinositol hydrolysis. If this hypothesis

[2] J. Chen, C.E. Protzman, and D.F. Woodward, unpublished observations.

is correct, then one might expect that the other receptors that stimulate cyclase (DP and IP) would be in the same group with Hup-4 and that the determinants of selective ligand binding would involve only a few amino acids.

Another feature of the phylogeny is the separate branch for the EP_3 receptors. As stated previously, extensive alternative mRNA splicing, yielding up to 10 isoforms of the EP_3 receptor, has been reported (15–18). To date, such alternative splicing has not been shown for the other EP, FP, or TP receptors, and this seems to be the case for Hup-4 as well; thus, both low stringency screening and PCR analysis have not yielded obvious splice variants (data not shown). This suggests that the alternative mRNA splicing giving rise to EP_3 receptor heterogeneity evolved uniquely and after the initial divergence of the EP_3 receptor from the other PG receptors. PCR analysis, however, suggests that an intron that has been noted in the genomic structure of the EP_3 and TP receptors (17) is conserved in the Hup-4 gene (see above). This intron, whose location corresponds to transmembrane region 6 in the receptor, appears to be present in members of both of the major branches of the PG family, which may indicate that it will be found in all PG receptor genes. mRNA splice variants giving rise to receptor isoforms, however, have not been observed for this intron.

The present results indicate that Hup-4, a human placenta cDNA, encodes the pharmacologically defined EP_2 PG receptor subtype. First, as expressed in COS-7 cells, the pharmacology of Hup-4 is consistent with the known EP_2 receptor pharmacology. Second, as expected for an EP_2 receptor, activation of Hup-4 by PGs stimulates cAMP formation. Third, Hup-4 mRNA is present in tissues that have been defined as containing EP_2 receptors. Although previously cloned receptors have been designated as EP_2 (12, 13), the EP_2-selective agonist butaprost did not compete with [^{3}H]PGE_2 binding to those receptors. With Hup-4, however, butaprost did compete and stimulated the formation of cAMP. Hup-4 and the previously cloned EP receptors, therefore, share a similar pharmacology and the ability to stimulate adenylyl cyclase but are clearly distinct EP receptor subtypes, sharing only ~30% overall amino acid identity. Identification of Hup-4 as a cDNA encoding the EP_2 subtype suggests that the previously cloned EP_2 receptors may represent the EP_4 subtype. Further studies of these cloned receptor subtypes will help to clarify the EP receptor classification and facilitate the development of subtype-selective drugs.

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